Effects of Post-Castration Interval Length on Recovery of Epididymal Sperm of Canine and Equine Sperm

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Introduction

Within male animals, spermatazoa are stored in the epididymis. The epididymis is part of the testicle that is just above the testis. The purpose of the epididymis is to mature, concentrate, transport, and store spermatozoa. It is tube shaped and attaches to the ductus deferens. Three distinct regions make up the epididymis: the head, the body, and the tail. The head's purpose is to initiate the physiological changes that need to occur for the spermatozoa to be a mature sperm cell. The body then transports the mature sperm to the tail, or cauda, where it is stored. In cases of sudden death, epididymal sperm can be extracted from a castrated animal. Electroejaculation is an inconsistent technique because of urine contamination in the semen (Viera, 2013.). As an alternative, the technique of cauda epididymal aspiration has developed to obtain epididymal sperm. In this process, the ductus deferens and cauda epididymis is dissected away from the rest of the testicle and then flushed with a certain type of extender fluid. The sperm is extracted during the flush. This technique is particularly useful in animals with larger testicles, like horse. In animals with smaller testicles, a more crude method of simply mincing the epididymis can be employed to gather viable sperm. In cases of untimely death, the animal can be castrated and semen can be extracted from the epididymis and then frozen for later use. Studies have shown that cryopreservation and subsequent thawing of dog semen results in no significant decrease ins sperm quality (Hewitt, 1999). This is an especially useful procedure for sires with valuable genetics that have died unexpectedly. This technique has been employed in numerous species including horse (Viera, 2013), goats (Blash, 2000), pigs (Ikeda, 2001) and even Spanish Ibex (Fernández-Santos , 2011). The procedure can also be used when young animals, like colts, are castrated before they show valuable mature genetics. One can also see how the technique can be of vital importance when seeking to preserve endangered wild species. The technique should be implemented in a variety of contingency plans in numerous production or zoo settings. Some studies have shown that interval between castration and flushing may have an effect on the quality of the sperm collection. The aim of this study was to observe any discernible changes in the quality of collected epididymal sperm as the interval between castration and collection increases, so as to provide a recommendation for the proper time to collect epididymal sperm.

Materials and Methods

Materials

- Equi-tainer
- Gloves
- Conical Tubes
- 25 mL syringe
- 3 mL syringe
- 22.5 gauge needle

- Compound microscope
- Dissecting Dish
- Forceps/Scissors
- E-Z Mixin 2-way Equine semen extender
- E-Z Freezin “LE” Lactose EDTA
- EQUIPRO CryoGuard Yolk-based Cryoprotectant
- BoviPure Percoll Top Layer
- BoviPure Percoll Bottom Layer
- Centrifuge
- Slides/cover slips
- Hemocytometer
- Counter
- Microtubes
- Heat Sealer
- Liquid Nitrogen
- Freezing straws
- Straw Freezing Kit
- Freezing chamber
- Thawing chamber
- Cooling boat (rack)
- 1 mL pipet
- 5-200 microliter pipet
- Wire piece

Procedure for Equine

1 Stallions will be castrated, and testicles placed in glove and ductus deferens tied off. Store in Equi-tainer, so as to cool testicles down to five degrees Celsius.
2 Begin dissection no later than 48 hours post castration.
3 Place castrated testicle in dissection dish.
4 The tunica albuginea should be first cut away from the testicle
5 Locate the epididymis and ductus deferens, and using scissors and forceps remove the epididymis from the main testes.
6 Using scissors, cut epididymal head and body away from the tail, leaving tail attached to ductus deferens.
7 Then carefully trim connective tissue from convoluted tubes as much as possible to straighten epididymal tail.
    a This will be tedious and take some time, but the more that can be straightened, the better the recovery of semen will be.
    b If it dries out too much to effectively cut, you can add a drop of E-Z Mixin extender.

c If the tube breaks before reaching the advanced convolutions, immediately flush it (See Step 8-12).

8 Attach 22.5 gauge needle to syringe and fill 25 mL syringe with at least 10 mL with extender(E-Z Mixin).

9 After the epididymis can no longer continue to be dissected due to break in tubule, separate smallest end of epididymis from the remainder of the cauda and place into conical tube labeled “First Flush”.

10 Using forceps, or partner using hands, hold ductus deferens vertical and use forceps to keep open the opening.

11 Insert the syringe into the vas deferens.

a Be careful not to allow needle to stick through the sides, and to hold everything exactly vertical.

12 Gradually apply pressure on the plunger end of the syringe and continue flushing until swelling appears in epididymis or pressure causes fluid to flow out of the top of the ductus deferens.

a The sperm should now be collected in the conical tube.

13 Place collecting tube in centrifuge for five minutes at 400G (1900 RPM).

a Be sure to fix a counter weight and check the RPM on the chart.

14 After centrifugation, remove supernatant using disposable pipet being careful not to affect sperm pellet.

15 Reconstitute pellet with 1mL of semen extender (E-Z Mixin).

a Vigorously tap bottom of tube to reconstitute.

b Use pipet if necessary to break up pellet, but eject from pipet gently.

16 Prepare warmed (room temperature) Percoll gradient tube (1mL bottom, 1mL upper). Gently pipet sperm on top layer. Centrifuge for 20 minutes at 400G.

17 Separate supernatant carefully using pipet.

18 Add with 1 mL of cryoprotectant extender (E-Z Freezin) and reconstitute as described previously.

a Add more if concentration is so high you cannot possibly count it, but be careful not to add too much.

19 Prepare a 1:20 solution of sperm sample:water by preparing a microtube with 95 microliters of water with 5 microliters of sperm sample,

20 Then apply 5 microliters of semen to the hemocytometer. Place small drop (around 1microliter) on hemocytometer slide.

21 Use hemocytometer to determine concentration and rate motility by eye using high power microscope and a counter by counting the sperm in five squares,

a Only count the sperm that are on the top and left border-lines, not those on the right and bottom border-lines.

b The total number of sperm will be in millions (250 sperm=250 million sperm per milliliter).

22 Calculate to find how much cryoprotectant extender to add to reach concentration of 200 million sperm per milliliter.

a Regardless, add 1 mL of cryoprotectant extender to sperm sample.

23 Label freezing straws, and prepare freezing chamber (Styrofoam box and liquid nitrogen, with rack outside of box).
   a Use protective goggles and gloves
   b Be sure there is enough liquid nitrogen to fill the bottom and float the rack.
24 Using straw-filling apparatus, load semen and then take up air until semen is touching cotton plug, leaving enough room at the tip for sealing.
   a Once semen touches the cotton, that end is sealed and you can remove it from the syringe
25 Seal straws with sealing machine. Seal both sides of the non-cotton end; flip it over and reseal in the same spot. Check to ensure complete sealing.
26 Load straws onto freezing rack, then place rack 3-6 cm above liquid nitrogen (which is what the rack will be set up to do anyway) and return top to the freezing chamber.
27 Allow five minutes for straws to reach -120 degrees Celsius. Then remove top and knock off straws so they plunge into liquid nitrogen.
28 Prepare thawing chamber (Styrofoam box filled with room temperature water, about 20 degrees C).
29 Thaw semen by using forceps to retrieve straw and place in thawing chamber. Thaw for ten minutes, then remove from water and cut the sealed tip (without cotton).
30 Use wire device to push semen out into mini tube for further analysis by pushing on the cotton end.

Procedure for canine performed as with equine except with the following modifications:

1. The canine epididymis is much smaller than the equine epididymis so it is not feasible to flush it, instead mincing should be done.
2. To mince, use the foreceps and scissors to tear apart the dissected epididymis as much as possible in the dissection dish.
3. Using a pipet, moisten the mince epididymis with at least 1 mL of extender.
4. Liquid should pool at the bottom of the dish.
5. Pipet the liquid at the bottom of the dish. This is the extender with the minced sperm (it is okay if you pipet some blood/tissue).
6. Place conical tube in centrifuge for five minutes at 400G (1900 RPM).
   a. Be sure to fix a counter weight and check the RPM on the chart.
7. After centrifugation, remove supernatant using disposable pipet being careful not to affect sperm pellet.
8. Reconstitute pellet with 1mL of semen extender (E-Z Mixin).
   a. Vigorously tap bottom of tube to reconstitute.
   b. Use pipet if necessary to break up pellet, but eject from pipet gently.
9. Prepare warmed (room temperature) Percoll gradient tube (1mL bottom, 1mL upper). Gently pipet sperm on top layer. Centrifuge for 20 minutes at 400G.
10. Separate supernatant carefully using pipet.
11. Add 1mL of EQUIPRO CryoGuard to the sample and reconstitute using the same tapping method.

12. Place in refrigerator for 2 hours so that sample reaches proper temperature before freezing.
13. After 2 hours, label freezing straws, and prepare freezing chamber (Styrofoam box and liquid nitrogen, with rack outside of box).
    a. Freezing, storage, and thawing to be conducted the same way as equine.

Motility Test

1. Prepare thawing chamber (Styrofoam box filled with room temperature water, about 20 degrees C).
2. Thaw semen by using forceps to retrieve straw and place in thawing chamber. Thaw for ten minutes, then remove from water and cut the sealed tip (without cotton).
3. Use wire device to push semen out into mini tube by pushing on the cotton end.
4. Place 8 µl drop of the sample onto a glass slide and place a cover slip on top.
5. Place the slide on the compound microscope.
6. Uses the microscope to view the sperm and make a rough estimate of the motile sperm out 100 (Spermvision software is unable to be used due to the yolk-based cryoprotectant).

Morphology Test

1. Thaw the sperm using the same method as previously described.
2. Prepare a 1:20 solution of sperm sample:water by preparing a microtube with 95 microliters of water with 5 microliters of sperm sample,
3. Then apply 5 µl of semen to the hemocytometer. Place small drop (around 1 µl) on hemocytometer slide.
4. Use the counter to count the morphologically abnormal sperm out 20 cells in 5 different frames.
5. The final number should be recorded as morphologically abnormal sperm out 100 cells.

Pope Stain: used to examine if there is damage to the acrosome cap

Preparing the Pope Stain Media

1. Weigh out 1.0 g of Rose Bengal into a weigh boat.
2. Weigh out 1.0 g of Fast Green FCF into a weigh boat.
3. Carefully place both of the Rose Bengal and Fast Green FCF into a 250mL Erlenmeyer flask.
4. Under a hood, add 60mL of PBS to the Erlenmeyer flask.
5. Then add 40mL of absolute ethanol (200 proof) to the Erlenmeyer flask.
6. Carefully swirl the flask, mixing the contents within the flask.
7. Vacuum filter the solution into a sealable container. Label the container as Pope Stain, and include the date and your initials.
8. This stain should be stored in a sealed container at room temperature.

Procedure for Staining Sample

1. Prepare the staining area with the following supplies:

a. Paper towels
b. Latex gloves
c. Sealable container of Pope Stain
d. Centrifuge tube holder
e. Unused centrifuge tubes
f. 4.0 µl pipette
g. 20.0 µl pipette
h. Unused pipette tips
i. Unused microscope slides
j. Sharper marker

2. Place 200 µl of concentrated (undiluted) semen into a centrifuge tube. (If spinning multiple tubes at one time be sure to label tubes accordingly)
3. Spin the centrifuge tube(s) at 5000 rpm for 6 minutes.
4. Using a pipette, remove the supernatant from the centrifuge tube leaving just the pellet. Discard the supernatant into the trash.
5. Add 100 µl of PBS to the centrifuge tube and re-suspend the sperm using the Fisher Scientific Mini Vortexer machine.
6. Put on latex gloves to avoid getting the stain on your hands.
7. Place 4.0 µl of re-suspended semen into an unused centrifuge tube.
8. Add 20.0 µl of the Pope stain to the same centrifuge tube.
9. Repeat this for all samples.
10. Let centrifuge tubes sit for at least 70 seconds.
11. While waiting, label the end of each slide with "Pope Stain", whether it is sexed or conventional semen, the date and your initials.
12. After 70 seconds, pipette 20.0 µl of the semen/stain mixture onto a slide and smear.
13. Place slide under the hood to air-dry for a couple minutes before observing under a microscope.

Observing the smeared slide under a microsope

1. Set up a microscope to observe the prepared slide. Use the Olympus LH50A microscope.
2. Place the slide on the stage of the microscope.
3. Turn on the microscope.
4. Adjust the eyepiece so that you can look at the sample with both eyes. Adjust your chair to appropriate height to minimize neck strain.
5. Locate the sperm cells using the 10x objective by adjusting both the coarse adjustment knob and the fine adjustment knob.
6. Proceed by locating the sperm cells using the 20x objective and then the 40x objective.
7. Using a counting device, count the first 100 sperm you see moving across the field; sperm will either be considered to have an intact acrosomal cap or one that has begun to/fully detach(ed).
8. Record your results as not fluorescing sperm out of 100.
9. Turn off and unplug the microscope. Return to the lowest objective and cover in order to protect the microscope from possible damage.
10. Slides can then be discarded into a glass disposal box.

Acridine Orange Stain: used to determine if DNA is intact

Procedure for staining sample

1. Place PBS on warming plate, heat to 38° C so as to not "shock" the sperm.
2. Get straws.
3. Place each straw into a hot water bath set to 35° C and let thaw for 30 seconds.
4. Cut straws with scissors at both ends and allow the semen to empty into a micro-centrifuge tube tat is respectively labeled with the correct semen title.
5. Add 100 µl of heated PBS to contents of tube.
6. Mix by re-pipetting.
7. Place in centrifuge at 25 rpm for 5 minutes and make sure both tubes pellet well and that the centrifuge is balanced.
8. Take out of centrifuge an check for supernatant, then pull off supernatant by pipetting it out.
9. Now add 100 µl heated PBS to each tube.
10. Mix by re-pipetting.
11. Place in centrifuge and spin at 25 rpm for 5 minutes again. Be sure centrifuge is balanced.
12. Take out of centrifuge and check for supernatant, then pull of supernatant by pipetting it out.
13. Get out 5 slides (4 for the smears and 1 for feathering each smear) and place 4 onto a piece of paper towel and the 5^{th} one will feather the sample.
14. Pipette 25 µl from each tube and place onto its corresponding slide.
15. Use the edge of the 5^{th} slide to smear.
16. Allow all 4 slides to air dry for a minimum of 10 minutes.
17. Place all 4 slides into individual slots in the Carnoy's solution and leave for 3 hours. Secure closed with parafilm.
18. Put on gloves and remove slides from the Carnoy's solution and let slides dry for 15-20 minutes.
19. Place slides in 0.1M Citric Acid Solution and rinse slides thoroughly with DI water 4 times.
20. Place Acridine Orange solution for 5 minutes and secure with parafilm.
21. Rinse slides with DI water 4 times.
22. Let slides air dry on a paper towel with the sperm sample side facing up in the dark for 10 minutes.
23. Place cover slips on slides and let set for 1 minute.
24. Seal edges of cover slips with clear nail polish.
25. View the slides under fluorescent microscope and take pictures if necessary.

Results

The post-castration intervals that were tested were 1 hour, 8 hours, 25 hours, 30 hours and 48 hours. Motility of the sperm gathered from the canine testicles decreased significantly at the 25 hour interval mark. Morphology, acrosome integrity, and DNA integrity were generally unaffected by the interval length. Morphology was measured as a percent of abnormal sperm out

of 100. Acrosome was measured as a percent of sperm cells with compromised acrosome integrity out of 100. DNA integrity was measured using the Acridine Orange stain fluorescence and was determined on a high/low basis. The mean motility across all intervals was 38%. The mean abnormal morphology was 37% (63% normal). The mean level of sperm cells with compromised acrosome integrity was 15% (85% with good integrity). Three yearling colts were also castrated and analyzed but no sperm was discovered in the epididymis. Table 1 describes the the overall dat of the analyzed samples. Figure 1 demonstrates the descending trend of motility decreases as the interval length increases. Motility was highest after 1 hour post-castration and lowest after 25 hours and 48 hours post castration. Figure 2 demonstrates the inconsistency observed in the level of acrosome integrity as interval length increased. Acrosome integrity was the lowest after 48 hours and highest after 25 hours. Figure 3 demonstrates the percent of abnormal sperm as the interval increased. Abnormal morphology was the highest after 30 hours and lowest after 25 hours. Figures 4-7 show the fluorescence of different samples. Green fluorescence indicates that the DNA within the sperm is intact. Figures 8-13 show the samples at various points during collection and analysis. Figures 14-16 show the lack of sperm found in samples collected from yearling colts.

Table 1. Overview of collected data

Sample	Species	Age	Time of Castration	Time of Collection
1	Canine (Weimaraner)	2 years	5/1/2013, 11:00 AM	5/2/13, 5:00 PM
2	Canine (Weimaraner)	2 years	5/1/2013, 11:00 AM	5/3/2013, 11:00 AM
3	Canine (German Shephard)	2 years	4/12/2013, 8:00 AM	4/12/13, 4:00 PM
4	Canine (Pitbull)	Indeterminant	5/23/13, 12:00 PM	5/24/13, 1:00 PM
5	Canine (Corgi)	Indeterminant	5/24/13, 12:00 PM	5/24/13, 1:00 PM
6	Equine (Quarterhorse)	Indeterminant	2/22/13, 12:00 PM	2/22/13, 1:00 PM

Sample	Interval Length, Hours	Motility (%)	Morphology (%)	Acridine Orange	Pope Stain
1	30	10.00	32%	High	28%
2	48	1.00	57%	High	1%
3	8	70.00	32%	High	4%
4	25	1.00	22%	High	30%
5	1	80.00	36%	High (70%)	25%
6	25	50.00	56%	High (90%)	24%

Figure 1. Percent Motile as post-castration interval increases

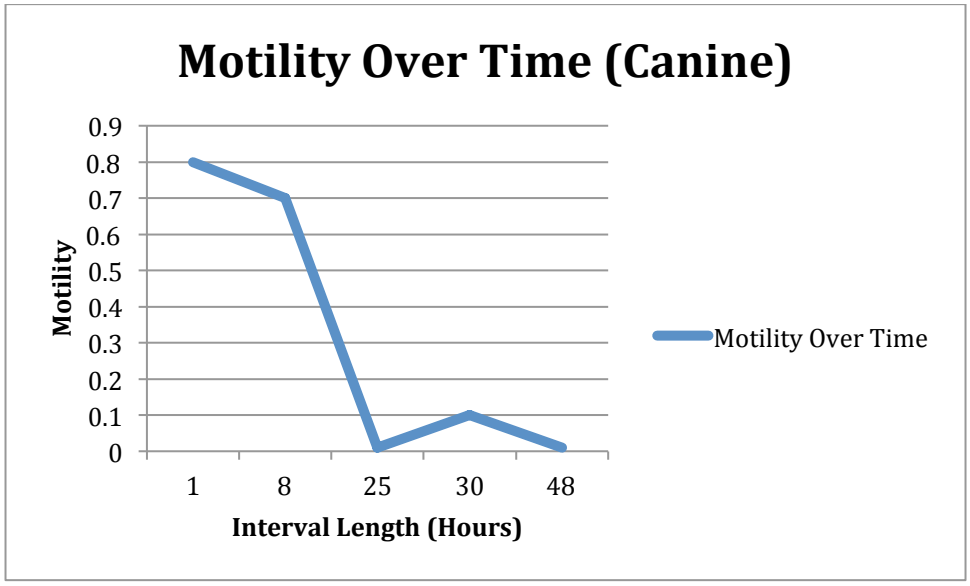


Figure 2. Acrosome integrity measured by Pope staining as post-castration interval increases

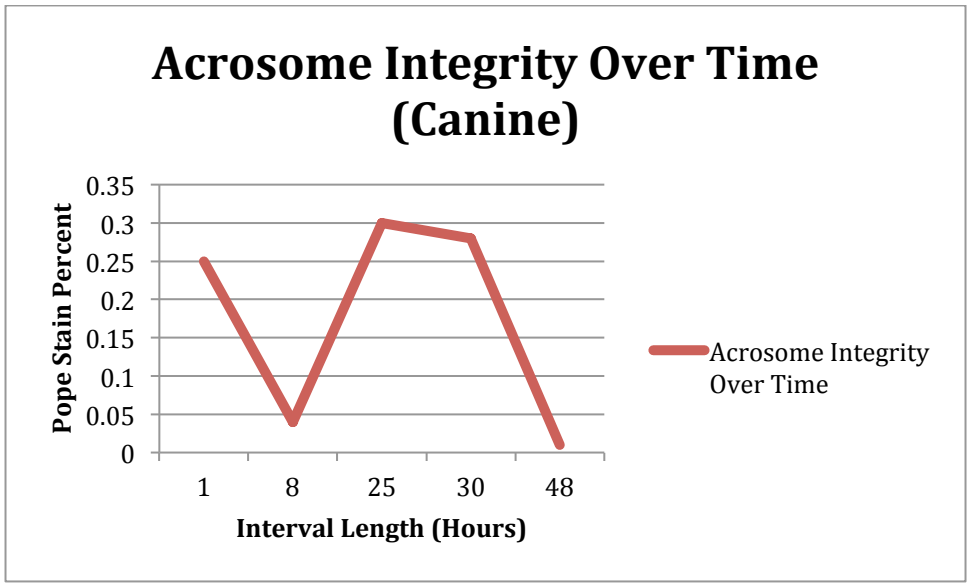


Figure 3. Abnormal Morphology Percent as Post-Castration Interval Increases

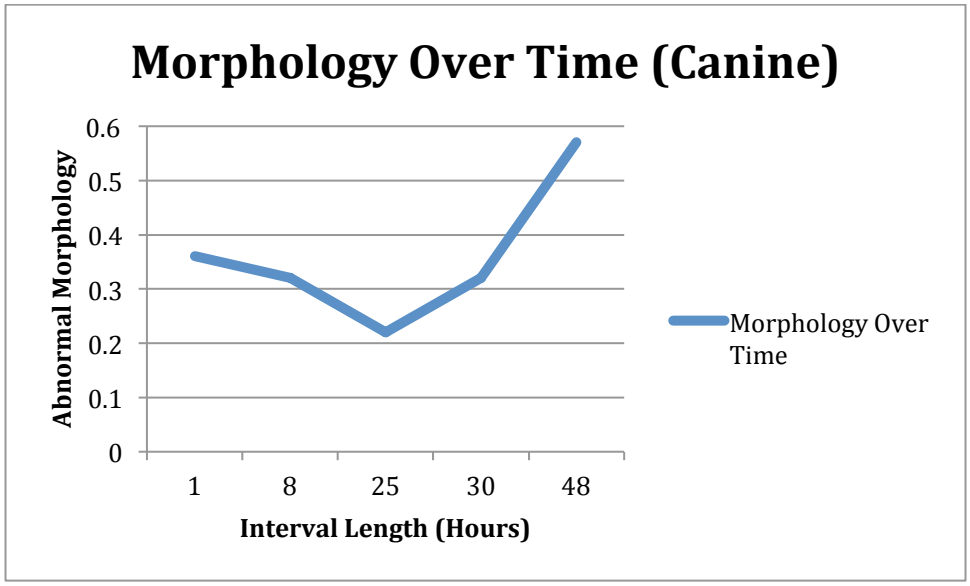


Figure 4. Sample 3 Acridine Orange Stain

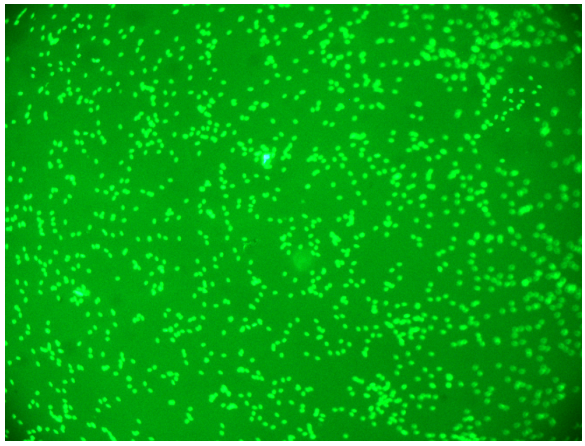


Figure 5. Sample 4 Acridine Orange Stain

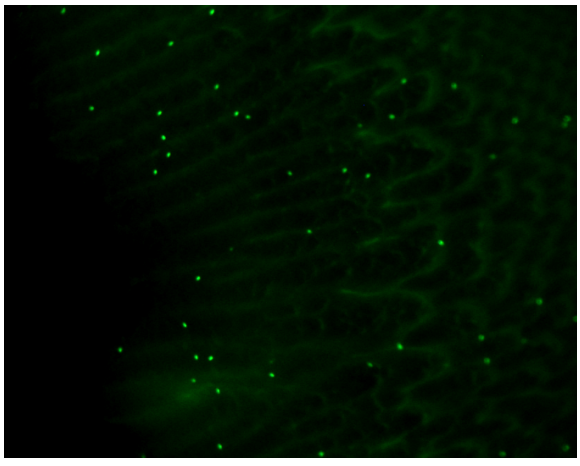


Figure 6. Sample 5 Acridine Orange Stain

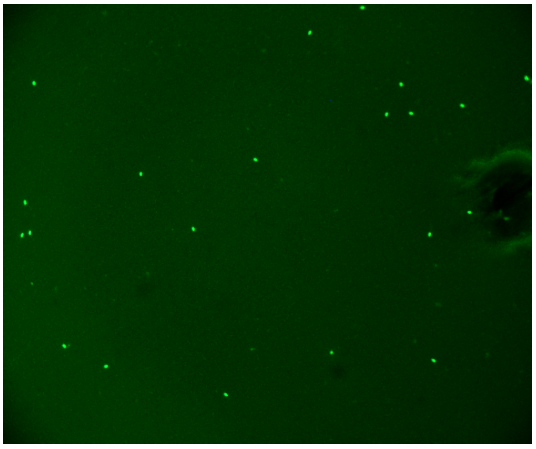


Figure 7. Sample 6 Acridine Orange Stain

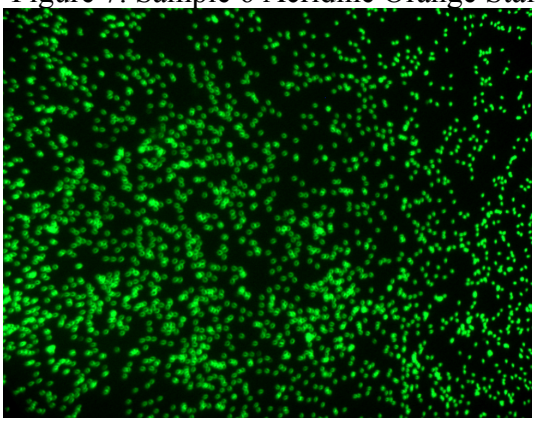


Figure 8. Sample 1. After Initial Collection

Figure 9. Sample 2 with Cryoprotectant

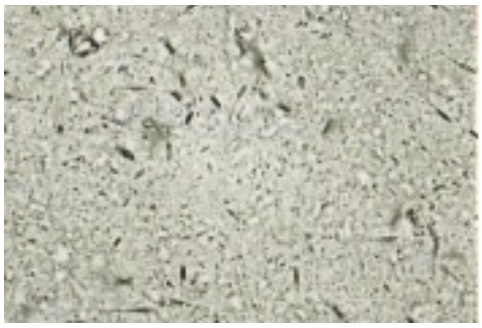


Figure 10. Sample 3 After Initial Collection

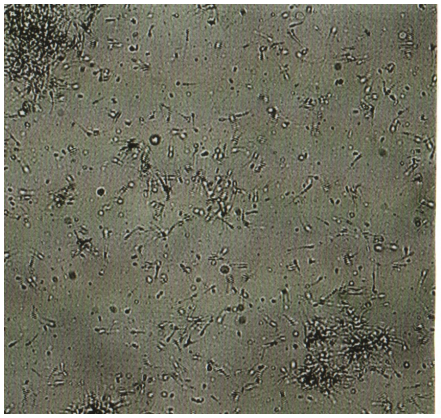


Figure 11. Sample 4 After Initial Collection

Figure 12. Sample 5 After Reconstitution

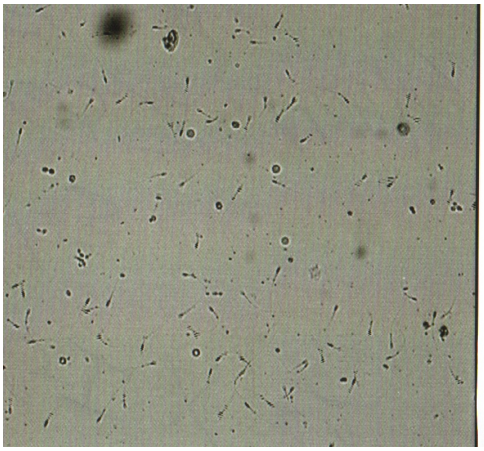


Figure 13. Sample 6 Post Thaw

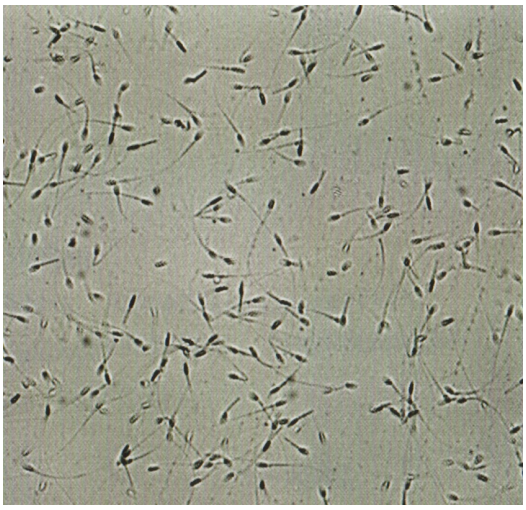


Figure 14. Yearling colt castrated 5/8/13 (No result)

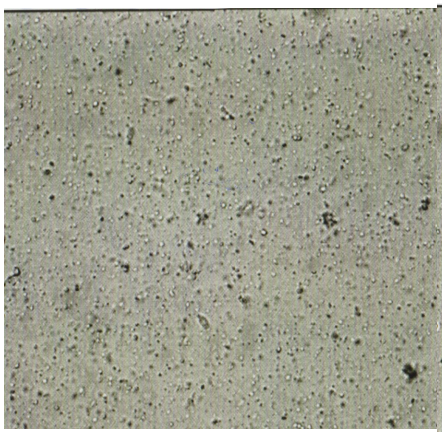


Figure 15. Yearling colt castrated 5/10/13 (No result)

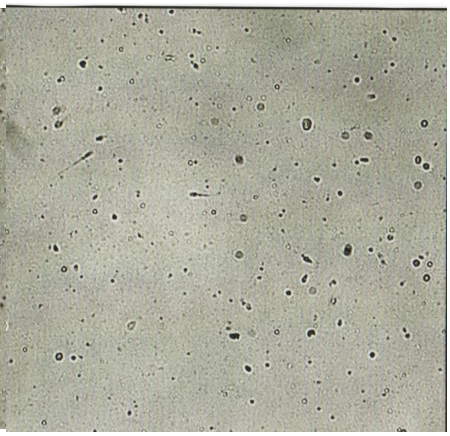
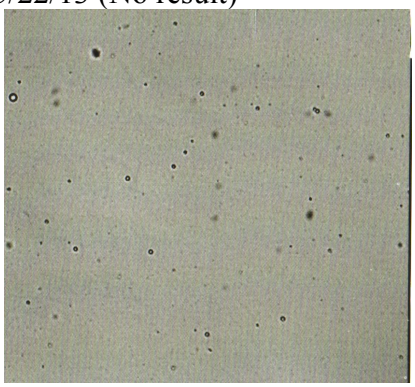


Figure 16. Yearling colt castrated 5/22/13 (No result)



Discussion

Artificial insemination is a valuable tool employed by dog and horse breeders alike. In the event of untimely death of genetically valuable sire, sperm can still be recovered and used if the animal is castrated and collected within a particular window of opportunity. Numerous studies have confirmed that the viability of sperm decreases as the post-castration interval increases (Viera, 2013). With the horse, studies have also shown that sperm can remain viable for as long as 96 hours after castration (Viera, 2013). In the present study, methods were used to recovery sperm from the epididymis of canines for 1 hour, 8 hours, 25 hours, 30 hours and 48 hours after castration had occurred. One sample was collected from a stallion after 25 hours since castration. Due to the epididymis of canines being extremely small and convultued the technique of simply mincing the epididymis was used to extract the sperm. In the equine sample, the technique of aspiration of the cauda epididymis and ductus deferens was used to extract the sperm.

The longer the interval between castration and collection exists the lower the quality of the recovered sperm will be. It is generally accepted that around 70% motility is needed for canine sperm to be used for reproduction success, This figures had been found to be 60% in horses (Rouge, 2003). Using these figures, reproductive success could only be possible attained with Sample 3 and Sample 5. Thus, the results gathered from this study indicate that proper time to perform post-castration epididymal sperm recovery would be some time during the first 8 hours after castration. The relative inconsistencies witnesses in the morphology, acrosome integrity and DNA integrity tests are expected because these are factors that would not be influenced by the post-castration interval. Motility would decrease as the sperm die as time goes on but abnormalities in morphology, acrosome integrity and DNA integrity are structural and would have occurred during development of the spermatozoon. The maintenance of DNA integrity has also been confirmed in similar studies with Spanish ibexes (Fernández-Santos, 2011). As long as the DNA remains intact, reproductive success can still be achieved through intra-cytoplasmic sperm injection (ICSI).

This study can be improved upon in several ways. First and foremost, a much larger sample size would be necessary to be sure of more accurate results. Of course the sample size is always limited by the accessibility of materials and in this study the most limiting factor would be the access to fresh testicles. The lack of testicles resulted in the poor results of the equine aspect of this study. Of the four horses that were examined, only one was a mature stallion with enough sperm to discern any meaningful data. This study has shown that in order to perform epididymal recovery of sperm in the horse, the animal should be older than 12 months, preferably at least 2 years. The experiment could also be improved upon through more accurate methods of assessment. All of the data (motility, morphology, DNA/acrosome integrity) was collected through counting by the eye and using a manual counter. This type of assessment is highly subjective and must acknowledge the presence of error by the individual analyst. For further research, fertility trials could be completed using in-vitro or artificial insemination techniques with the collected sperm. Any morphological abnormalities of the embryo could also be noted, so as to determine is post-castration epididymal sperm recovery is a viable technique to be used in animal reproduction. Pre-incubation has also been suggested as a possible mode of increasing reproductive success of epididymal sperm (Ikeda, 2001).

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